REMARKS

Applicants submit this Amendment in response to the Office Action mailed on April 23, 2008.

The claims have been amended as follows. Claim 48 has been amended for clarification, as discussed in more detail below. The amendment to claim 48 does not change the scope of this claim. Claims 49 to 51 have been added. Support for the new claims is found in several places in the specification, such as on pages 7 to 10 which discloses that pepSUAM (SEQ ID NO: 4) is the amino terminal portion of SUAM (SEQ ID NO: 15). The amino acid sequence of SEQ ID NO: 4 is that of amino acids 66 to 80 of SEQ ID NO: 15.

Rejections of the Claims

I. Rejection of claim 48 under 35 U.S.C. §112, first paragraph

The Examiner has rejected claim 48 under 35 U.S.C. §112, first paragraph, for indefiniteness because of the use of the transitional limitation "having". Applicants traverse the rejection of this claim on this ground.

Applicants submit that there is no indefiniteness in the claim as presented prior to this Amendment. It is clear from the claim that the polypeptide has an amino acid sequence that consists of SEQ ID NO: 4. However, in order to facilitate prosecution of this application, Applicants have amended claim 48 to remove the transitional limitation "having". The scope of the claims is not affected by this amendment.

Applicants respectfully request the Examiner to withdraw the rejection of claim 48 on the ground of indefiniteness under 35 U.S.C. §112, first paragraph.

II. Rejection of the Claims under 35 U.S.C. §103

The Examiner has rejected claims 1, 2, and 48 under 35 U.S.C. §103(a) as being obvious in view of the combined disclosures of Park, in Proceedings of the 40th Annual Meeting of National Mastitis Council, National Council Incorporated, pages 247-248, February 2001; or Fang, FEMS Microbiol. Lett. 176:91-96 (1999); in view of Hammerschmidt, Infect. Immun. 67:1683-1687 (1999); Staggs, Mol. Microbiol. 12:613-619 (1994); or Biswas, Infect. Immun. 67:455-459 (1999). Applicants traverse the rejection of these claims on this ground.

The disclosures of Park and Fang, as indicated by the Examiner, are similar although not identical. Both Park and Fang disclose a lactoferrin-binding protein that is isolated from *Streptococcus uberis* along with other surface proteins of this microorganism. Five strains of *S. uberis* were employed in the study by Fang, and four of the five strains contained a 112 kDa protein that was identified as a lactoferrin-binding protein. Four strains of *S. uberis* were employed in the study by Park, and all of these four strains contained a 112 kDa protein that was identified as a lactoferrin-binding protein. Both studies demonstrated binding of *S. uberis* to bovine lactoferrin using rabbit anti-bovine lactoferrin in a protein-protein binding blot.

The Hammerschmidt, Staggs, and Biswas references disclose methods of purifying bacterial lactoferrin-binding proteins. It is the Examiner's contention that, the disclosure of Park or Fang of an isolated but not purified bacterial lactoferrin binding protein together with the disclosure of Hammerschmidt, Staggs, or Biswas of methods to purify bacterial lactoferrin-binding proteins indicates that the present invention of a novel lactoferrin binding protein that was purified from the isolated mixture of proteins of Park or Fang is obvious. Applicants respectfully disagree.

Prior to the present application, one lactoferrin-binding protein ("LBP") of *S. uberis* was known. This LBP was disclosed in Jiang, WO 98/21231, which reference was submitted as Reference BA in an Information Disclosure Statement of March 8, 2004. The disclosure of Jiang, WO 98/21231, is similar to Moshynskyy et al, Microbial Pathogenesis, 35:203-215 (2003), which article was available online at http://www.sciencedirect.com/ as of September 13, 2003, prior to the filing date of the present application. The Moshynskyy article is submitted herewith.

Jiang discloses the discovery that *S. uberis* produces a lactoferrin-binding protein. The amino acid sequence of the LBP of Jiang is disclosed in SEQ ID NO: 2. On page 25, first paragraph, Jiang discloses that *S. uberis* produces two protein species that are able to bind lactoferrin. One of these has a molecular weight of 165 kDa and the other has a molecular weight of 76 kDa. Data provided below in Jiang establishes that these two proteins are the same and that the 165 kDa protein is a dimer of the 76 kDa protein. Thus, only a single protein of *S. uberis* is disclosed in Jiang to be capable of binding lactoferrin. The fact that the 165 kDa and 76 kDa proteins represented the same proteins but in various states of dimerization was established by denaturing the proteins with urea, which resulted in elimination of both 165 and 76 kDa bands and the appearance of a single band of about 105 kDa. See Jiang, page 60, lines 17-32. Additionally, Jiang determined the nucleotide sequence of the LBP gene and determined that a protein that would be encoded by this gene would have a molecular weight of about 62 kDa, which predicted size was disclosed to be comparable to the 76 kDa molecular weight monomer protein.

From page 66, line 27, to page 69, line 33, Jiang tested 47 strains of *S. uberis*, including 5 strains obtained from the ATCC (American Type Culture Collection) and 42 field isolates to determine the presence of the LBP in these strains. The results are shown in Table 2 on pages 68 and 69. Jiang determined that, although there was variation in the N terminal portion of the LBP in the various strains, each of the strains had the LBP and the C terminal portion was highly conserved in all of the strains. It is noted that one of the strains listed in Table 2 of Jiang is ATCC 13387. The significance of this is discussed below.

The present application discloses a lactoferrin-binding protein of *S. uberis*. As stated on pages 5-6, bridging paragraph of the specification, the term "*Streptococcus uberis* Adhesion Molecule," which is also referred to by the acronym "SUAM," is utilized so as not to confuse the polypeptide of the present invention with the lactoferrin-binding protein of Jiang et al., WO 98/21231, which is a different protein.

Prior to the present application, only a single lactoferrin-binding protein of *S. uberis* was known, that disclosed in Jiang, WO 98/21231. Both Park and Fang disclose that a mixture of proteins was isolated from *S. uberis*, which mixture was subjected to denaturing (SDS) PAGE and produced lactoferrin-binding proteins of apparent size of 110 and 112 kDa, which size is almost identical to the 105 kDa of the denatured LBP of Jiang. Therefore, one skilled in the art would expect that the LBP proteins included in the isolated mixture of Park or Fang were the same as the LBP protein disclosed in Jiang.

This expectation would be heightened by the fact that one of the strains utilized in Park and Fang was ATCC 13387. Both Park and Fang disclose that this strain was utilized and disclose that it contained the 112 kDa band lactoferrin-binding protein. Because Jiang discloses

that the ATCC 13387 strain produced the LBP of Jiang and does not disclose the presence of additional lactoferrin-binding proteins in ATCC 13387, one skilled in the art would conclude that the lactoferrin-binding protein of Jiang and the lactoferrin-binding proteins in the mixture of Park and Fang were the same.

The comments made by the Examiner in the present Office Action support this contention that one skilled in the art, at the time of isolation of the protein mixture as reported in Park and Fang, would expect that the identified proteins were the same as that of Jiang. On page 4 of the present Office Action, the Examiner states the following regarding the cited Park reference:

> Because of the overlapping molecular weight, the *Streptococcus uberis* origin of the prior art polypeptide, the identical *Streptococcus uberis* ATCC13387 strain from which it was extracted by the identical SDS extraction method, the prior art polypeptide is viewed as the same as the isolated polypeptide in the instant claims, and therefore it is expected to have the same intrinsic structure and properties as that of the Applicants' polypeptide.

On pages 4-5 of the present Office Action, the Examiner makes a similar comment regarding the disclosure of the cited Fang reference.

At the time prior to the present invention, it is submitted that there was no motivation to purify the lactoferrin-binding proteins of Park and Fang because one skilled in the art would expect that the isolated protein of Park and Fang would be the same as that of Jiang, which protein had already been purified and sequenced. However, as disclosed in the present specification in Example 6 on pages 25-26, Applicants did purify their protein and, as disclosed

in Examples 7 on pages 26-27 and Example 15 on pages 41-44, determined the amino acid sequence of the pepSUAM and SUAM polypeptides.[1]

Purification and subsequent determination of the sequence provided the discovery of a novel polypeptide. Prior to this purification, and the analysis of the purified polypeptide, there was no suggestion that an LBP in *S. uberis* other than the LBP of Jiang existed. At this time, there was a simultaneous conception and reduction to practice of the presently claimed invention. Prior to the purification, with subsequent sequencing, there was neither conception nor reduction to practice.

The cited secondary references, together with either or both the Park and Fang primary references, do not disclose or suggest the present invention. The Hammerschmidt, Staggs, or Biswas references are cited merely for their disclosures of methods that one skilled in the art may use to attempt to purify proteins from an isolated mixture of proteins. There is no suggestion in the secondary references, either alone or in combination with the primary references, of the novel SUAM polypeptide of the present application, the existence of which

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[1] To definitively confirm that SUAM and LBP (Jiang/Moshynskyy) are different proteins, several protein/DNA BLASTING alignments were conducted using software available at the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nig.gov). Results showed no significant similarities between SUAM and LBP at the amino acid and DNA levels. In addition, SUAM and LBP amino acid sequences were aligned using ClustlW2 (http://www.ebi.ac.uk/tools/clustalw2/index.html), a protein alignment tool. Results of this alignment yielded a very low score (11 out of 100), indicative of a lack of similarity between SUAM and LBP. The unique nature of the SUAM polypeptide of the present invention is further evidenced by the fact that when the N-terminal amino acid sequence was used to search the *S. uberis* genome for the SUAM nucleic acid sequence, reverse translation, there was only a single location and a single reading frame that coded for SUAM. The results show that only one reading frame (DNA to amino acid) provided a complete protein that included the pepSUAM sequence. In addition, western blot analysis using antibody to pepSUAM revealed the presence of pepSUAM in all *S. uberis* strains tested to date, indicating that the N- terminus of SUAM is highly conserved. This finding is in direct contrast to the disclosures of Jiang/Moshynskyy which report variation in the N- terminal portion of the LBP in the various strains tested. Taken together, these results definitively establish the unique and novel identity of the SUAM polypeptide disclosed in the present application. Applicants are not providing this information in a Declaration because the issue of the identity of SUAM and the differences between SUAM and LBP has not been raised by the Examiner. However, if the Examiner does raise this issue, or if a Declaration is felt to be necessary for any other reason, Applicants will submit such a Declaration.

polypeptide and its distinctness from the previously known lactoferrin-binding protein of *S. uberis* was discovered only upon subsequent purification and characterization as disclosed in the present application.

In addition, in at least one of the references cited by the Examiner, Staggs et al, Molecular Microbiology, 12(4):613-619 (1994), the purification method employed failed to result in a purification of a protein but rather yielded only an identification of the presence of a multiplicity of lactoferrin-binding proteins that were present in the subject microorganisms. It is respectfully submitted that this disclosure underscores the fact that purification of a particular protein of interest following isolation of a mixture of proteins that includes the particular protein of interest is not, as maintained by the Examiner, routine.

Applicants submit, therefore, that the presently claimed invention is not obvious as the presently claimed polypeptide was not suggested by the prior art and further represents an unexpected departure from the prior art which disclosed a single LBP of *S. uberis*. Accordingly, Applicants submit that the rejection of claims 1, 2, and 48 under 35 U.S.C. §103(a) for being obvious over the combined disclosures of Park or Fang in view of Hammerschmidt, Staggs, or Biswas, is overcome and respectfully request the Examiner to reconsider and to withdraw the rejection of the claims on this ground.

Additional Comments

Applicants point out that claim 48 has been amended to cancel the phrase "having an amino acid sequence." As stated in the present Office Action in section 9, deletion of this phrase would obviate the art rejection of record. Accordingly, in addition to the reasons

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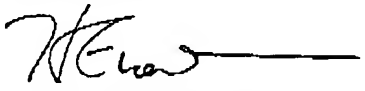
discussed above in relation to the art rejections of claims 1, 2, and 48, Applicants submit that the rejection of claim 48 as being obvious over the combined disclosures of Park or Fang in view of Hammerschmidt, Staggs, or Biswas, is overcome and respectfully request the Examiner to reconsider and to withdraw the rejection of claim 48 on this ground.

CONCLUSION

Applicants submit that the claims, as amended herein, are in condition for allowance and request an early notice to that effect.

Respectfully submitted,



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Attachment: Moshynskyy et al, Microbial Pathogenesis, 35:203-215 (2003)

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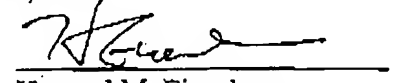

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# Characterization of a bovine lactoferrin binding protein of *Streptococcus uberis*

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**Abstract**

The interaction between *Streptococcus uberis* and bovine lactoferrin (bLf) has been characterized. The binding of $^{125}$I-bLf to *S. uberis* was time-dependent and displaceable by unlabeled bLf. The Scatchard plot was linear and approximately 7800 binding sites were expressed by each bacterial cell, with an affinity of $1.0 \times 10^{-7}$ M. Both heat and protease treatment of bacterial cells reduced bLf-binding significantly, indicating the presence of a cell surface localized protein receptor for the glycoprotein. One protein was identified from the cell wall of *S. uberis* as the functionally active bLf-binding protein and it existed in both monomeric and dimeric forms. The recombinant protein expressed in *E. coli* cells was able to bind bLf and had molecular weights similar to that of native *S. uberis*. Deletion analysis located the bLf-binding domain to a 200 amino acid region at the amino terminus of Lbp. Analysis of the primary and secondary structure suggested that Lbp is an M-like protein. An isogenic mutant of *S. uberis* lacking the internal sequence of the *lbp* gene was constructed by allele replacement. Adherence experiments with wild type *S. uberis* and the *lbp* mutant revealed that Lbp is not responsible for attachment of *S. uberis* to host epithelial cells.


*Keywords:* *Streptococcus uberis*; Lactoferrin; Adherence; Bovine; Virulence

## 1. Introduction

Bovine lactoferrin (bLf) is a 92.1 kDa iron-binding glycoprotein found in milk, various biological secretions, and polymorphonuclear leukocytes (PMN) [1,2]. During acute bovine mastitis, the bLf concentration in lacteal secretions can increase up to 30-fold depending on severity of infection [3]. The biological roles of lactoferrin (Lf) include amplification of the inflammatory response by promoting adhesion and aggregation of PMNs to the endothelial surface [4], stimulation of the phagocytic and cytotoxic properties of macrophages [5] and the regulation of myelopoiesis and thereby antibody production by feedback inhibition of granulocyte–monocyte colony-stimulating factor [6].

Lactoferrin can exert a powerful inhibitory effect on bacterial growth. Iron chelation and the resulting iron limitation for bacteria have been suggested to constitute one mechanism of Lf-mediated antimicrobial action [7]. Many Gram-negative pathogens, notably *Haemophilus influenzae* and *Neisseria meningitidis*, are capable of using iron-binding glycoproteins such as Lf or transferrin (Tf) as iron sources [8], and iron acquisition is mediated by bacterial surface receptors which specifically interact with the iron-binding glycoproteins [9]. Therefore, it is believed that the ability to compete with Lf and Tf for iron is essential for the pathogenesis of these bacterial infections, and that the receptors for iron-binding glycoproteins are important virulence factors. Some Gram-positive bacteria such as *Staphylococcus aureus*, coagulase-negative staphylococci and *Streptococcus agalactiae* also bind Lf through specific receptors on their surface [10,11]. However, in contrast to our knowledge of the iron uptake systems of Gram-negative bacteria, there is comparatively little information concerning the role of Lf receptors of Gram-positive bacteria in the uptake of glycoprotein-bound iron or their potential role in pathogenesis.

*Streptococcus uberis* is an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in both lactating and non-lactating dairy

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cattle [12,13]. Several potential virulence factors have been identified from *S. uberis*, including hyaluronic acid capsule [14], hyaluronidase [15], uberis factor, believed to be similar to CAMP factor of *S. agalactiae* [16], R-like protein [17], and a plasminogen-activating protein [18]. In the present study, we investigated the interaction of bLf with *S. uberis*, identified a specific bLf-binding protein (Lbp), and created an isogenic mutant unable to express a functional Lbp. The mutant was shown to adhere as efficiently as the wild type parent strain to MAC-T cells, indicating that attachment is not mediated by Lbp in the strain used for these studies.

## 2. Results

### 2.1. Binding of bLf to S. uberis

*S. uberis* strain Su-1 was tested for its ability to bind $^{125}$I-labeled bLf. The results showed that $^{125}$I-bLf was bound by *S. uberis* in a time-dependant manner with approximately 90 min required for 100% saturation (data not shown). A competitive binding experiment using bLf (33% iron-saturated) as both radioligand and competitor was performed. Unlabeled bLf effectively displaced the binding of $^{125}$I-bLf to *S. uberis* in a concentration-dependent manner (Fig. 1), indicating that the interaction between *S. uberis* and bLf was specific. A concentration of approximately 279 nM of unlabeled bLf resulted in 50% blocking of $^{125}$I-bLf uptake. A Scatchard plot [19] was linear, suggesting that one component of the cell wall was responsible for bLf binding and that *S. uberis* bound approximately 7800 molecules per cell with an affinity (Kd) of $1.0 \times 10^{-7}$ M.

To determine whether the degree of bLf iron saturation could influence receptor binding, apo-bLf was used as a competitor in the $^{125}$I-bLf binding assay. The data presented in Table 1 shows that apo-bLf and iron-saturated bLf had the same binding receptor on the *S. uberis* cell.

The specificity of binding was determined by using bTf, human lactoferrin (hLf) and human transferrin (hTf) to inhibit the binding of $^{125}$I-bLf to *S. uberis* cells. None of these proteins interfered with the binding of $^{125}$I-bLf to *S. uberis* (Table 1), suggesting that the binding was bLf-specific.

In order to determine if the bLf-binding property of *S. uberis* was mediated by an iron-regulated bacterial component, EDDA, dipyridyl or deferroxamine mesylate were incorporated in THB-YE broth to reduce the availability of free iron. Cells prepared following growth in these iron-restricted conditions did not show increased $^{125}$I-bLf binding relative to cells grown under iron replete culture conditions.

### 2.2. Characterization of a cell wall bLf-binding protein

Treatment of *S. uberis* cells with pepsin, trypsin and proteinase K abolished bLf-binding, indicating the involvement of a surface-exposed cell wall protein(s) (Table 1).

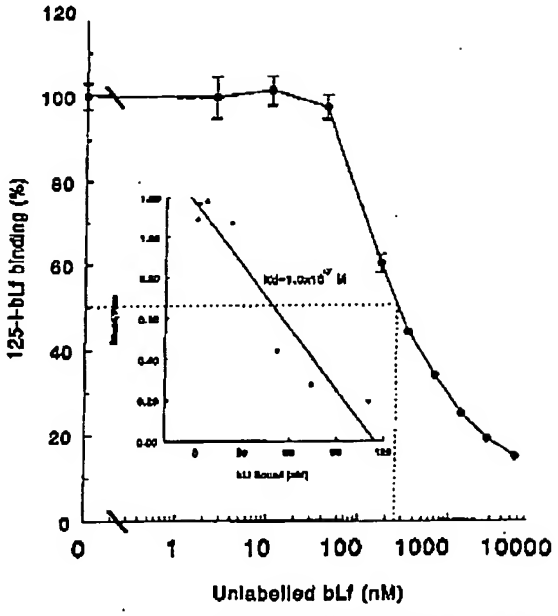


Fig. 1. Competition binding assay using 33% saturated bLf as radiolabeled ligand and competitor. Binding values were calculated as the percentage of $^{125}$I-bLf binding in the presence of increasing amount of unlabeled bLf relative to samples with no unlabeled bLf. Inset: Scatchard plot (on the basis of one experiment; another experiment showed a similar result) and affinity (Kd) of the binding of $^{125}$I-bLf to *S. uberis* [19]. The line represents the best fit as determined by a linear regression analysis. A concentration of 270 nM of unlabeled bLf caused 50% displacement of $^{125}$I-bLf binding (indicated by dotted lines).

Table 1
Inhibitory effects of unlabelled proteins and enzymes or heat treatment of bacteria on $^{125}$I-bLf binding to *S. uberis* strain Su-1

| Inhibitor or cell treatment | Mean percentage inhibition[a] or decrease[b] in binding |
|---|---|
| *Inhibitor* | |
| bLf | 94.6 (0.1) |
| apo-bLf | 94.7 (0.2) |
| bTf | 2.5 (1.4) |
| hLf | −14.0 (2.4) |
| hTf | 1.9 (1.3) |
| *Proteases* | |
| Pepsin | 85.7 (3.7) |
| Trypsin | 89.1 (4.4) |
| Proteinase K | 92.7 (2.4) |
| *Heating* | |
| 50 °C | −4.3 (6.0) |
| 80 °C | 29.6 (2.1) |
| 100 °C | 65.6 (4.8) |

[a] Inhibition values were calculated as relative percentage of bLf binding to bacteria suspended in PBS in the absence of any inhibitor. Figures in parentheses are standard deviations.

[b] Decrease in binding were calculated as relative percentage of bLf binding to bacteria without any treatment.

This proteinaceous component was somewhat heat-labile, since heat treatment of bacteria reduced bLf-binding. The presence of the functionally active Lbp in a cell wall preparation of *S. uberis* was demonstrated by Western blotting using $^{125}$I-bLf (Fig. 2). Under non-reducing conditions, two components with apparent molecular weights of 165 and 76 kDa were identified as Lbps (Fig. 2, lane 1). Under reducing conditions, the binding of bLf was significantly reduced. The binding of bLf by these two protein bands was specific, since the presence of unlabeled bLf effectively blocked the binding (data not shown).

### 2.3. Cloning and expression of the lbp gene

A gene library was constructed in pTZ18R with chromosomal DNA from *S. uberis* strain Su-1. Approximately 5000 transformants were screened for their ability to bind bLF by colony blotting with $^{125}$I-bLf, and a total of seven colonies showing detectable signals were selected for further study. Whole cell lysates from these recombinants were further tested for their ability to bind $^{125}$I-bLf under non-reducing conditions. The clone with the strongest signal, designated DH5α (pLBP5), generated three major bands in blotting experiments. Two of the reactive bands had molecular weights of 165 and 76 kDa (Fig. 2, lane 3), similar in size to those of *S. uberis* (Fig. 2, lane 1). A third band, slightly larger than 165 kDa, was most likely a precursor form with an uncleaved signal peptide. Although not evident in the figure, the 76 kDa protein is often seen as a doublet. No corresponding band was found from the whole cell lysate of DH5α (pTZ18R), the host strain control (Fig. 2, lane 2). The Lbp(s) was also detected in periplasmic and supernatant extracts of DH5α (pLBP5) (Fig. 2, lanes 6 and 7, respectively), but not in outer or inner membrane extracts (Fig. 2, lanes 4 and 5, respectively), indicating that

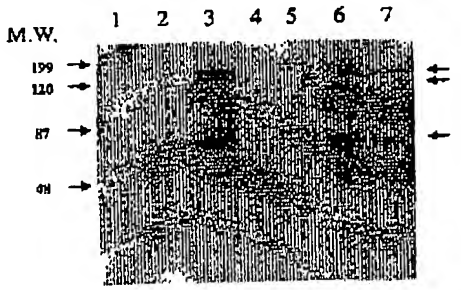


Fig. 2. Expression of bLf-binding proteins by recombinant *E. coli* pLBP5. All samples were separated under non-reducing conditions by 7.5% SDS-PAGE followed by blotting to a nitrocellulose membrane which was probed with $^{125}$I-labeled bLf. Lane 1, cell wall of *S. uberis*; Lane 2, whole cell lysate of *E. coli* pTZ18R; Lane 3, whole cell lysate of *E. coli* pLBP5; Lane 4, outer membrane of *E. coli* pLBP5; Lane 5, inner membrane of *E. coli* pLBP5; Lane 6, periplasm of *E. coli* pLBP5; Lane 7, supernatant of *E. coli* pLBP5. The molecular mass standards (M.W., kDa) are indicated by arrows to the left, the $^{125}$I-bLf-reactive bands are indicated by arrows to the right.

the protein(s) expressed in *Escherichia coli* DH5α was not membrane-localized, but instead was secreted from the cell.

To determine if the protein with a molecular weight of 165 kDa was a dimer of the 76 kDa molecule, samples treated with 2-mercaptoethanol or urea were analyzed by Western blotting using rabbit antiserum against the recombinant Lbp protein. Mercaptoethanol treatment had no effect on the electrophoretic mobility of the proteins from either *S. uberis* or recombinant *E. coli*, while urea-treatment resulted in disappearance of the 165 and 76 kDa bands and appearance of a new band with an apparent molecular weight of 105 kDa (data not shown). This suggests that the 165 kDa protein was a dimer of the 76 kDa subunit.

### 2.4. Nucleotide sequence determination and analysis

Sequence analysis of the insert in the plasmid pLBP5 revealed the existence of two open reading frames (ORFs; Fig. 3). One ORF encoded Lbp, since its presence in subclone pLBP5L resulted in a bLf-binding phenotype (Fig. 4). The second ORF on the complementary DNA strand was incomplete. A search of the GenBank database showed that the presumed gene product of the second ORF had significant homology to the Mga (VirR and Mry) transcriptional regulators of group A streptococci (GAS). Therefore, this incomplete ORF was named *mga* (Fig. 4).

The *lbp* sequence contained two potential translational ATG start codons at positions 232 and 262 of the DNA sequence. Both were associated with putative Shine-Dalgarno sequences (Fig. 3). These start sites resulted in predicted protein products of 62,857 and 61,454 kDa, respectively. We have no evidence to indicate which ATG is used.

The DNA sequence shows two putative −10 and −35 promoter regions present at nucleotides −88 and −102 from the first ATG. Downstream of *lbp* there is a potential *rho*-independent transcription terminator (Fig. 3).

Analysis of the predicted N-terminal sequence of Lbp revealed a putative secretion signal sequence [20] (Fig. 3) with a positively charged domain rich in K and R residues, followed by a hydrophobic domain from amino acid 25 to 48. A signal peptidase cleavage site, VKA, at position 49 to 50 was also present. The presence of this putative signal sequence indicates that Lbp is likely exported across the cytoplasmic membrane of *S. uberis* and is consistent with the surface exposure of the protein.

A search of the GenBank database revealed that the C-terminus of Lbp was homologous to the C-terminal ends of streptococcal M proteins, as well as streptococcal plasminogen-, fibrinogen- and IgG-binding proteins. Lbp exhibits all the general features well established for surface proteins of Gram-positive cocci [21], including a cluster of four charged amino acids at the C-terminus, followed by a hydrophobic domain of 21 amino acids (Fig. 3). Adjacent to the hydrophobic domain is a consensus membrane anchor motif LPSTGD. The next region of 50 amino acids is the cell

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TTATTTATTAATACGAGAAAACCTGTTTCCTTTTGGAGGAACCAGGAAATAATCTCAATC  60
                                                    -35
TAGCATTATATAGTGATAAAAACTCATTTATCATTCATTATTTTAAATAAAAATTAAAAA 120
   -35      -10           -10
GTTGAGCTTTATTATCACGATAAACTAATATATACTAATTTTGTAGTTTCGAGAAGCCCT 180
                                    SD
TTTAGCGTGTCTAAAACGTTATCATTTCAGCTTTTTGCAAAAACTAACAAAATGAGAAAA 240
                                                    M  R  K    3
TTCTATTATAAGGAGAAGAAAATGGAAATCAAACAAAAACATGGCAAGCATGCTTTACGC 300
  F  Y  Y  K  E  K  K  M  E  I  K  Q  K  H  G  K  H  A  L  R   23
AAAGCGGTCACCGCAGCGGTCTTAGCAGGGACAGCTTTCTCAAGCTTAGGAGGCTTCGCA 360
  K  A  V  T  A  A  V  L  A  G  T  A  F  S  S  L  G  G  F  A   43
GGAGCAGTAACAACAGTCAAGGCGGAAGAATTTACGGATAAATATTATCCTAAAATGAAT 420
  G  A  V  T  T  V  K  A  E  E  F  T  D  K  Y  Y  P  K  M  N   63
AAAATGGATGAAGAGGATCTTTTGCGATTAAGCGCTGAAAGCAAAAAGGACGTTCCTGCA 480
  K  M  D  E  E  D  L  L  R  L  S  A  E  S  K  K  D  V  P  A   83
ATACGAGAATTATTAAAAAGAATGACTACGGAATCAATACATGCTTTATTAGTGGGACTA 540
  I  R  E  L  L  K  R  M  T  T  E  S  I  H  A  L  L  V  G  L  103
GATTCTTCCCACATTTCTTATAGTGAAGAGAGTGGGTTCAATAATTTGCTTAGAAAATTT 600
  D  S  S  H  I  S  Y  S  E  E  S  G  F  N  N  L  L  R  K  F  123
GGGTCTATGAATAATGACGACCCTTCAGATTGGACTCATTATAAAAGTGGTGTTAGTTTA 660
  G  S  M  N  N  D  D  P  S  D  W  T  H  Y  K  S  G  V  S  L  143
GCTTCTATAATTGTCCAAGAAGTAAGAAGTAGATTGAATGAAAAAGATGAACTTGATGAA 720
  A  S  I  I  V  Q  E  V  R  S  R  L  N  E  K  D  E  L  D  E  163
          └→A1
GAACTATCTAACAAGAAAGAAGAACTTCAGAAACTAACTGAAAAAATAGAAAAAACAATT 780
  E  L  S  N  K  K  E  E  L  Q  K  L  T  E  K  I  E  K  T  I  183
AAAGAAAAAGAGAATTTAAACAAAGAAATCACGGAAAAAAATTCTGAAATTTCTAAAATG 840
  K  E  K  E  N  L  N  K  E  I  T  E  K  N  S  E  I  S  K  M  203
                                          A1←┘  └→B1
GAAGAGGAACTTAGTGAAAAAGAAAAAGAAATTGCAGAAAATAAAGAAGAACTAGCAGAT 900
  E  E  E  L  S  E  K  E  K  E  I  A  E  N  K  E  E  L  A  D  223
                   B1←┘  └→C1
GCTTTAGGTGAACTTTTTGATGCTGAGGAAACAATTGATAAAAAAGAAGCTAAAGTTAAA 960
  A  L  G  E  L  F  D  A  E  E  T  I  D  K  K  E  A  K  V  K  243
GATTTAACTGAAAAATTAGATGCTTCTAGAAAAGAACATGAGGCACTTGCTAAAGAGTTT 1020
  D  L  T  E  K  L  D  A  S  R  K  E  H  E  A  L  A  K  E  F  263
GCAGAGTCTCAAAAAGGTTATGAAAAAGAGTTAGCTGATAAACACACTGCTTTAGGTGAA 1080
  A  E  S  Q  K  G  Y  E  K  E  L  A  D  K  H  T  A  L  G  E  283
                C1←┘                                    └→A2
GCTGAAAAACGTAATGCTGATTTAGAGGCTGGCAACAAAGAACTTAAAGAAAACTTAGAA 1140
  A  E  K  R  N  A  D  L  E  A  G  N  K  E  L  K  E  N  L  E  303
ATGGCTGAAGGTATCTCTGATGACTTGCAGAAGAAAGTCATGAAAGCAGAACAAGAGATG 1200
  M  A  E  G  I  S  D  D  L  Q  K  K  V  M  K  A  E  Q  E  M  323
AAAGAACTTTCTGCACAATTAGAAGAAGCAAAAGAAGAACTTGAAACTGAAAAAGCTAAG 1260
  K  E  L  S  A  Q  L  E  E  A  K  E  E  L  E  T  E  K  A  K  343
  A2←┘  └→B2                                  B2←┘  └→C2
```

Fig. 3. Nucleotide and deduced amino acid sequences of Lbp. Nucleotides and amino acids are numbered on the right of the sequences. The predicted amino acid sequence is shown directly below the nucleotide sequence. Two possible ATG start codons at positions 232 and 262, and the TAA stop codon at 1915 are shown in bold type. Two putative −35 and −10 promoter sequences and Shine-Dalgarno sequences (SD) are indicated and a putative rho-independent transcription terminator (T) is underlined. The double underline shows the presence of a putative signal peptide at the N-terminus of the ORF. The C-terminal hydrophobic transmembrane domain is indicated by italics and the nearby surface anchor motif is shown by italic and double underline. The central repeat amino acid sequences are indicated by letter A, B and C.

```
TTAGCAGAGTCAGAAAAAGAAAATGCAAAACTGACCGAAGAACGTGATGCTGCTAAGAAA 1320
  L  A  E  S  E  K  E  N  A  K  L  T  E  E  R  D  A  A  K  K  363
GAAGCTGAAAAAGTTCCTGAACTAGAAGAGCAAGTTGAAAAATTAGTTGAAGAAATCACT 1380
  E  A  E  K  V  P  E  L  E  E  Q  V  E  K  L  V  E  E  I  T  383
GCTGCTAAGAAAGAAGCAGAAGAGCTTCAAGCTAAAGCCGAAGGCCTTGAAAAAGACTTT 1440
  A  A  K  K  E  A  E  E  L  Q  A  K  A  E  G  L  E  K  D  F  403
                                    C2←┘
GAAGCTGTTAAAGCAGAAAAAGAAGCCCTTGAAGCTGAAATTGCTAAATTGAAAGAAGAC 1500
  E  A  V  K  A  E  K  E  A  L  E  A  E  I  A  K  L  K  E  D  423
CACCAAAAAGAAGTGGACGCTCTCAATGCACTCCTTGCTGATAAAGAGAAAATGCTTAAG 1560
  H  Q  K  E  V  D  A  L  N  A  L  L  A  D  K  E  K  M  L  K  443
AACTTGCAAGACCAGCTTGACAAAGCTAAAGAAGAAGCTATGAAGAACGAGCAAATGAGC 1620
  N  L  Q  D  Q  L  D  K  A  K  E  E  A  M  K  N  E  Q  M  S  463

CAAGAAGAAAAAGCTAAATTGCAAGCTGAATTGGATCAAGCTAAGAAAGAATTGGCAGAA 1680
  Q  E  E  K  A  K  L  Q  A  E  L  D  Q  A  K  K  E  L  A  E  483
AAAATCAAAGACATGCCAAACAAAGTGGCTCCTCAAGCCGAAGGCAAAGCCAATGCAGGT 1740
  K  I  K  D  M  P  N  K  V  A  P  Q  A  E  G  K  A  N  A  G  503
CAAGCAGCTCCAAACCAAAACCAAAACAACCAAGCACAAGCAAATCAAGCTAAGAACGGC 1800
  Q  A  A  P  N  Q  N  Q  N  N  Q  A  Q  A  N  Q  A  K  N  G  523
AACAACCTCCCATCAACAGGTGACAAACCAGTTAACCCACTCCTAGTGGCAAGTGGTCTC 1860
  N  N  L  P  S  T  G  D  K  P  V  N  P  L  L  V  A  S  G  L  543
TCCCTCATGATCGGAGCAGGTGCCTTCGTCTACGCCGGCAAACGCAAAAAAGGCTAAGTC 1920
  S  L  M  I  G  A  G  A  F  V  Y  A  G  K  R  K  K  G        561
ATAACAAAGGACTTCCAGAAACTTTCCTAAAAAATTATTTTCTATTCATACTAAGACAGA 1980

AAACTCCCCCTACCATGAGGTAAGGGGAGTTTTATTACACTTAAGGATAAATAGGCAACT 2040
      T                 T
CTGGGAAGAGGGCTTGTAAAATTTTAGGGGTGATGGCTTGGCCTGACCCTTCCTGACAGT 2100
```

Fig. 3 (*continued*)

wall-associated region characterized by a high proportion of proline and glycine residues (12%).

The region upstream of the membrane anchor motif and proline/glycine rich region contained three blocks of repeated amino acids (Fig. 3). Analysis of the secondary structure of the translated protein showed an extensive alpha-helical region stretching from the proline/glycine-rich region to the beta-sheet and turn region near the cleavage site of the signal sequence. This structure is typical of M-like proteins, contributing to dimer formation through a coiled-coil interaction.

### 2.5. Localization of the Lf-binding domain of Lbp

A series of gene deletions were constructed at the 3′ and 5′ ends of *lbp* in order to localize the region(s) responsible for binding of bLf (Fig. 4). The 3′ deletions present in plasmids pTP31, pTP32, pTP33 and pTP34 were generated at the restriction enzyme sites *HincII*, *StuI*, *XbaI* and *XmnI* of pLBP5, respectively, and a single 5′ deletion in pTP51 was constructed by removing the 643 bp *HindIII–XbaI* fragment of pLBP5. All these deletions retained the *lbp* promoter. The resulting truncated Lbp proteins were produced in *E. coli*, separated by non-reducing SDS-PAGE, transferred to nitrocellulose and probed with specific anti-Lbp serum or $^{125}$I-bLf. Truncated proteins from *E. coli* containing plasmids pTP31, pTP32 and pTP33 were present in both monomeric and dimeric forms, both of which could bind bLf (Fig. 4). Proteins expressed by recombinant *E. coli* strains containing plasmids pTP34 or pTP51 did not bind bLF in Western blot experiments, suggesting that the primary binding domain of Lbp is localized in a 22 kDa N-terminal fragment.

### 2.6. Distribution of lbp and mga sequences in S. uberis strains

DNA from a collection of 47 *S. uberis* strains (Table 2) was used in Southern hybridization experiments in order to determine the distribution of *lbp*. A 1.5 kb *HindIII–HincII* fragment of *lbp* (LP1 in Fig. 4) was used as a probe. This probe hybridized with DNA from 42/47 strains (Fig. 5 and Table 1) indicating that most strains contained *lbp* sequences. The five strains, which did not contain DNA homologous to *lbp* were later identified as non-*S. uberis* streptococci (data not shown). In order to localize the homologous region(s) further, the hybridization experiment was repeated using probes from the 5′, central and 3′ regions

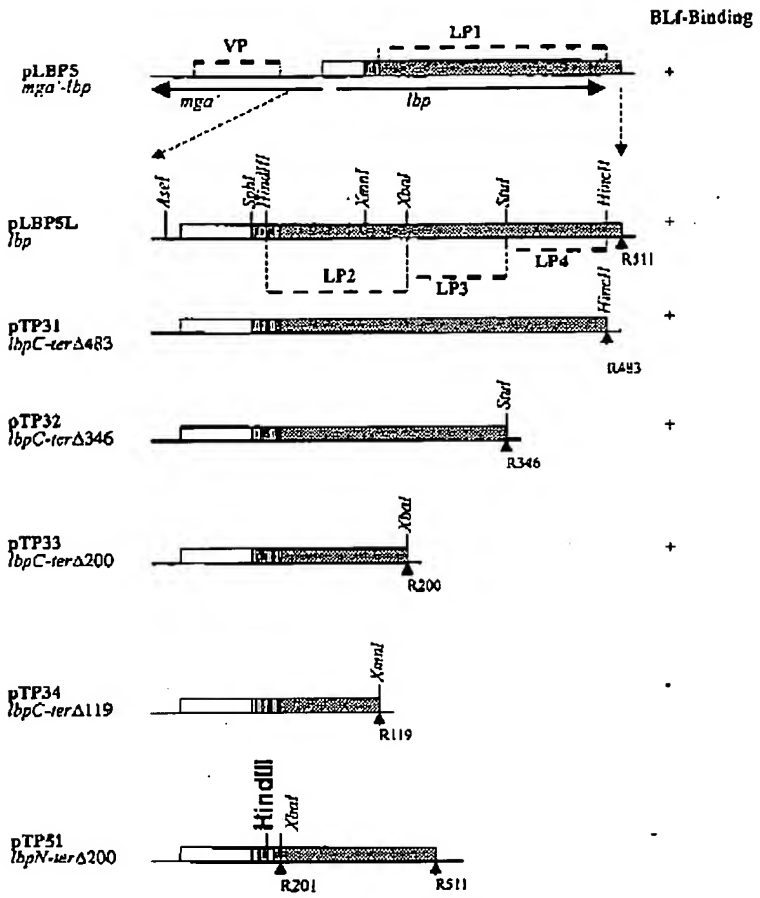


Fig. 4. Restriction map of *lbp* and bLf-binding by truncated Lbp constructs. Open boxes (p region) represent 5′ sequences containing the promoter and ribosome binding sites, shaded boxes (s region) represent the *lbp* signal sequence, and hatched boxes represent the *lbp* coding sequences. R1 and R201 indicate the first codon of the mature protein in pLBP5 and the truncated protein in pTP51, respectively. Other numbers indicate the last codon of each protein. The ability of each clone to bind bLf is indicated on the right. Probes used in hybridizations are indicated by LP1, LP2, LP3 and LP4.

of the gene, respectively (Fig. 3, probes LP2, LP3, LP4). The probes LP2 and LP3 covering the 5′ and central portion of the *lbp* gene, respectively, hybridized only with Su-1, the strain from which the *lbp* gene was originally cloned, whereas the 409 bp *StuI–HincII* fragment from the 3′ end of *lbp* (LP4) hybridized with all strains tested. Thus, the region of the *lbp* gene encompassing a portion of the C2 repeat, the proposed wall attachment region and the membrane anchor were conserved among *S. uberis* strains, while upstream regions were more variable.

In order to study whether there was an *mga*-related gene in the other *S. uberis* strains, Southern hybridizations were conducted using a 572 bp fragment (VP) that encompassed the 5′ region of the *mga* gene. Hybridization was detected in all *S. uberis* strains that showed homology with the *lbp* gene (Table 2).

### 2.7. Generation of an Lbp-deficient mutant

In order to determine the role of *lbp* in pathogenesis, a mutant was constructed in which the coding sequence was disrupted by the insertion of an *aad9* spectinomycin-resistance gene. Allele replacement was performed in two steps, the first being the isolation of primary recombinants in which the plasmid pMF113 was integrated into the chromosome at 37 °C (non permissive for plasmid replication). Southern blot hybridization revealed that the plasmid had integrated into the chromosome such that the wild type *lbp* gene was located downstream of the inserted plasmid sequence (data not shown). This strain, designated Su-3721, was cultured at 30 °C and a spectinomycin-resistant, erythromycin-sensitive derivative strain SuM13 was obtained. Southern blot analysis was performed and confirmed that the wild type *lbp* gene had

Table 2
*S. uberis* strains and DNA hybridization with LP and VP probes

| Strain | Source[a] | Probe | | | | |
|---|---|---|---|---|---|---|
| | | LP1 | LP2 | LP3 | LP4 | VP |
| Su-1 | ATCC9927 | 3.40[b] | 3.40 | 3.40 | 3.40 | 0.87 |
| Su-2 | ATCC13386[c] | –[d] | – | – | 3.60 | 3.60 |
| Su-3 | ATCC13387 | 3.60 | – | – | 3.60 | 3.60 |
| Su-4 | ATCC19436 | 2.70 | – | – | 2.7 | 1.7 |
| Su-5, Su-6 | Field isolates[e] | – | – | – | – | – |
| Su-9 | ATCC27958 | 3.80 | – | – | 3.80 | 0.87 |
| Su-45 | Field isolate[e] | – | – | – | – | – |
| *S. uberis* strains Su 7, 8, 10–44 and 46–48 | Field isolates | 2.90–5.30 | – | – | 2.90–5.30 | 0.87–5.00 |

[a] All strains are field isolates except five ATCC (American Type Culture Collection) strains.
[b] Size of hybridizing fragment (in kb).
[c] Further identified as *S. parauberis*.
[d] Lack of hybridization.
[e] Untypable streptococcal species.

been replaced by the deleted allele containing the spectinomycin-resistance gene (data not shown). Immunoblot analysis (Fig. 6b) was carried out using rabbit anti-Lbp serum and the 70 kDa Lbp band present in the wild type strain was missing in the mutant. This is supported by the observation that the mutant was unable to bind DIG-labeled bLf (Fig. 6c) in whole cell ELISA's.

### 2.8. Adhesion assays

Both wild type Su-1 and the *lbp* mutant were able to adhere to cultured MAC-T cells (data not shown) and to cells extracted from fresh mammary gland tissue. The number of adherent bacterial cells was estimated by microscopy as approximately 100 per 1 MAC-T cell, and did not differ in the mutant strain. Adherence was not affected by the addition of either bLf or anti-Lbp serum. Thus, Lbp does not appear to play a role in adherence of *S. uberis* to bovine mammary gland epithelial cells. In support of this, flow cytometry analysis failed to detect significant differences in the extent of adherence of Su-1 and the *lbp* mutant to MAC-T cells. Furthermore, latex beads coated with recombinant Lbp did not adhere to cells extracted from mammary gland tissue (data not shown).

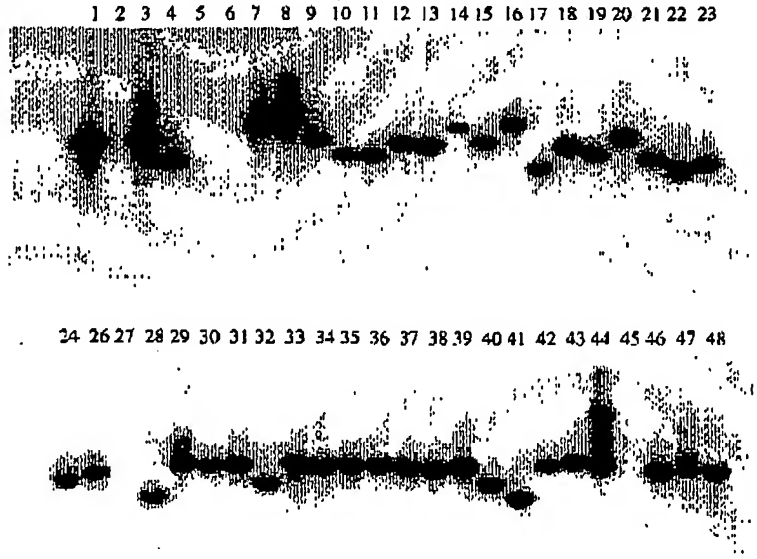


Fig. 5. Southern blot analysis of the *lbp* distribution in *S. uberis* strains. Chromosomal DNA from each *S. uberis* strain was digested with *HindIII* and probed with LP1 (see Fig. 3). *S. uberis* strain numbers are indicated at the top of each lane.

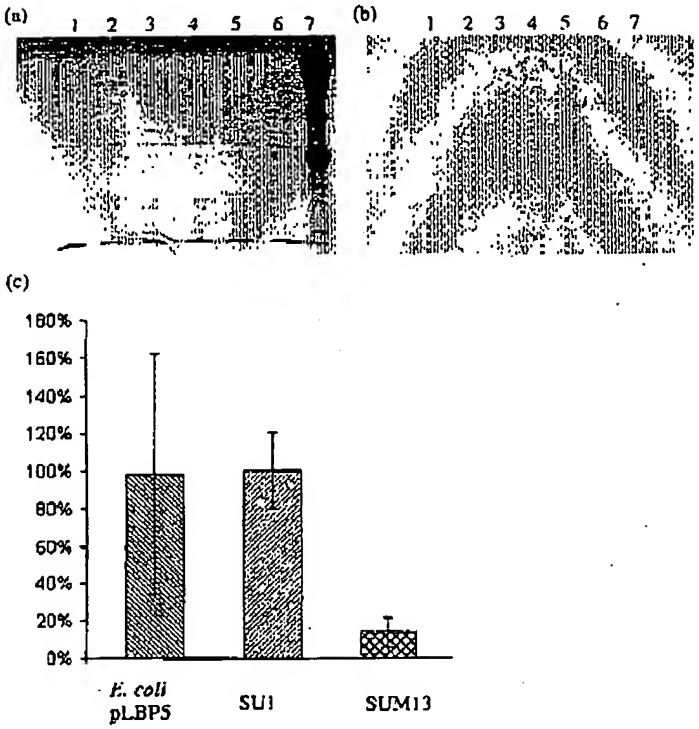


Fig. 6. Expression of Lbp by *S. uberis* Su-1 and its allele replacement derivatives. Total cellular proteins were separated by SDS-PAGE and blotted to nitrocellulose membranes as described above. Panel (a) represents coomassie blue-stained proteins, while panel (b) represents a Western blot. Lanes 1–4, Lbp$^-$ mutants; lane 5, single crossover recombinant (Su-3721); lane 6, strain Su-1. Panel (c) shows binding of DIG-labeled bLf by *E. coli* pLBP5, *S. uberis* Su-1 and SuM13.

## 3. Discussion and conclusions

Iron plays a central role in the pathogenesis of many bacterial infections, and numerous organisms have been shown to specifically bind iron-containing host glycoproteins as an iron-acquisition mechanism or to serve other virulence-related functions. We have demonstrated that the bovine pathogen, *S. uberis*, is able to bind bLf through the production of a novel surface protein, Lbp. *S. uberis* was able to bind both bovine apo-Lf and iron-saturated Lf, since both forms were equally effective in blocking $^{125}$I-bLf in competitive binding assays. The affinity of the *S. uberis* receptor for bLf ($1.0 \times 10^{-7}$ M) was slightly lower than that described for the bLf receptor of *S. aureus* ($7.1 \times 10^{-8}$) [10]. Lactoferrin from other species was unable to block the binding of bLf to Lbp, similar to the lactoferrin receptors of Gram-negative bacteria [22]. This is in contrast to the results described for *S. aureus* Lf receptors which could bind lactoferrins from both human and bovine sources [10,23]. Since *S. uberis* is strictly a bovine pathogen, while *S. aureus* can cause infection in both humans and cattle, the species-specificity of the receptor molecules is consistent with the host specificity of the two organisms. Neither bovine nor hTf was able to block the binding of bLf to *S. uberis*, similar to *S. aureus* [10]. The expression of most Tf and Lf receptors of Gram-negative bacteria such as *N. meningitidis* [22] and *H. influenzae* [24] is regulated by the amount of free iron in its environment. In contrast, the expression of the bLf-binding phenotype of *S. uberis* was not regulated by the availability of free iron in the growth medium. This is consistent with previously reported findings [11,23] which showed that the binding of Tf to *S. aureus* and Lf to *S. agalactiae* was not iron regulated. It is known that the Tf and Lf receptors of Gram-negative bacteria play an essential role in iron acquisition, whereas it appears that this is not the case for Gram-positive species.

Two Lbps with molecular weights of 165 and 76 kDa were identified by blotting experiments, where *S. uberis* components were probed with $^{125}$I-bLf. This was similar to the presence of two receptors coding for the transferrin-binding proteins of members of the Pasteurellaceae, but was inconsistent with the Scatchard plot analysis which suggested a single receptor. Treatment of *S. uberis* proteins with urea prior to SDS-PAGE resulted in a single protein with an apparent molecular weight of 105 kDa which was able to bind bLf. This suggested that the 165 kDa protein identified above may have been a dimer of the 76 kDa

molecule and that treatment with urea altered the electrophoretic mobility as has been observed in other species such as *N. meningitidis* [25]. Treatment of *S. uberis* with lower concentrations of urea did not alter the mobility of the 165 kDa band (data not shown) suggesting that the structure was very stable. Gene cloning experiments demonstrated that a single open reading frame was capable of producing two protein species capable of binding bLf. The DNA and predicted amino acid sequences showed significant homology to the streptococcal M proteins [26] as well as a number of alpha-helical coiled structure mammalian fibrillar proteins such as myosin heavy chain and kinesin heavy chain, suggesting a similar structure. This is consistent with the formation of dimers as is the case with the M protein family. The predicted amino acid sequence of the carboxy terminus of Lbp showed a typical cell wall anchoring motif found in many other surface proteins from Gram-positive bacteria [21,27]. Thus, we believe that Lbp is anchored to the *S. uberis* surface via this putative anchor region. This is supported by the observation that only the amino terminal 22 kDa of the protein was responsible for bLf-binding. However, the latter observation does not rule out the presence of repeated binding sites within the protein which would only be detected using a larger number of 5′- and 3′-deletions. As expected, the cell wall anchoring motif did not function in *E. coli* since the recombinant Lbp was found primarily in the periplasm and the growth medium, rather than associated with the membrane fraction. This is similar to the streptococcal M6.1 protein which was found predominantly in the periplasm of *E. coli* [28].

Southern blot analysis of *S. uberis* strains using *lbp* probes showed that while the 3′-portion of the gene appeared to be conserved among strains, the 5′-region showed greater variability. This result resembles those described for M protein genes in group A streptococci [29]. Since the 3′-end of the gene codes for a putative membrane anchor region, one would expect minimal sequence variation, whereas the portion of the gene coding for the amino terminal region would be variable due to immunological selective pressure. PCR analysis of the *lbp*-containing region of several *S. uberis* strains suggests that while flanking DNA sequences are similar, the internal sequences vary considerably (data not shown).

Interestingly, while sequencing the *lbp* gene we found a second, partial open reading frame encoding a protein of 499 amino acid residues. The predicted amino acid sequence had significant homology to the streptococcal VirR12 (499 residues) and Mry (530 residues) proteins, which are positive regulators of the M protein genes of group A streptococci [30]. This region was conserved amongst all *S. uberis* strains examined (data not shown), and it would be interesting to determine the role, if any, of the *S. uberis* Mga in the control of *lbp* expression.

Fang et al. [31] demonstrated that bLf or bovine milk promoted attachment of *S. uberis* to bovine mammary gland epithelial cells. These authors reported that three strains of *S. uberis* were found to be able to bind bLf, but purified Lf promoted adherence in only two of these strains. We were unable to demonstrate any enhancement of adherence following treatment of one *S. uberis* strain with bLf, and the isogenic Lbp-deficient mutant attached as efficiently as the wild type to both cultured mammary gland epithelial cells and fresh mammary gland tissue. Likewise, purified recombinant Lbp was unable to promote adherence of latex beads to mammary gland epithelial cells. Thus, Lbp does not appear to be responsible for the phenotype described by Fang et al. [31], suggesting either a different *S. uberis* component was responsible for bLf promoted adherence or that the strains used in the two studies were phenotypically different. Given the sequence diversity of the *lbp* region in *S. uberis*, we believe the later is a more plausible explanation. Based upon work done with Gram-negative pathogens, Lbp may also be involved in iron-acquisition. However, the protein was not essential for *S. uberis* cell growth, even in iron-depleted growth media. In addition, both the mutant and wild type strains incorporated similar levels of $^{59}Fe$ from bLf (data not shown), and therefore, we do not believe that it plays a role in iron acquisition. Lbp may function either as a signaling molecule or simply a means of promoting molecular mimicry through the binding of a host glycoprotein. These latter possibilities are the subject of further investigation.

## 4. Materials and methods

### 4.1. Bacterial strains, plasmids and culture conditions

The *S. uberis* strains used in this study are listed in Table 2. General cloning procedures were performed in pTZ18R [32], and allele replacement was carried out using the plasmid pG$^+$host 9 [33], a generous gift of Dr E. Maguin (INRA, France). *S. uberis* strain ATCC 9927, designated Su-1 in our laboratory collection, and derivatives were routinely cultured in Todd-Hewitt broth (Difco, Detroit, MA, USA) supplemented with 0.5% yeast extract (THB-YE), or in Brain Heart Infusion broth (BHI; Difco). *E. coli* strain DH5*alacIq*, was used as a host for plasmid DNA manipulations and gene expression experiments, and was cultured in Luria Bertani medium (LB; Difco). When necessary, media were supplemented with antibiotics (all from Sigma Chemical Co., St Louis, Missouri, USA) to the following concentrations: 50 μg/ml of ampicillin, 100 μg/ml of erythromycin, and 300 μg/ml of spectinomycin. Erythromycin and spectinomycin were used at the same concentrations for both *E. coli* and *S. uberis*. Bacteria were grown at 37 °C, unless otherwise specified.

### 4.2. Fractionation of bacterial cells

Cell wall components of *S. uberis* were extracted as described previously [34]. Twenty base #2 blood agar plates

were inoculated with *S. uberis* and incubated for 18 h at 37 °C. Bacterial cells were collected, washed once with 200 ml of 0.85% saline and resuspended in 50 ml of extraction buffer (0.05 M $Na_2HPO_4$, 0.15 M NaCl, 0.01 M EDTA, pH 7.4). Cell walls were extracted by shaking with glass beads (4 mm diameter) for 20 h at 37 °C. After centrifugation at 48,000*g* for 20 min, the supernatant (cell wall extract) was collected, filtered through a 0.22 μm membrane (Nalgene, Mississauga Ontario), dialyzed against distilled water, lyophilized and resuspended in 1 ml of distilled water. Outer and inner membranes were isolated from *E. coli* cells by sucrose density gradient centrifugation, as described elsewhere [35]. *E. coli* periplasmic proteins were prepared by the cold osmotic shock method described previously [36].

Whole cell lysates of *E. coli* transformants were prepared from cells washed once in 0.1 M phosphate-buffered saline (PBS), pH 7.2 and subjected to one freeze–thaw cycle. Thawed cells were pelleted by centrifugation at 6000*g* for 20 min and the supernatant was concentrated 100-fold by lyophilization. Concentrated culture supernatants from recombinant *E. coli* were prepared by precipitation with 10% trichloracetic acid (TCA). Pellets were resuspended in 0.01 volumes of 100 mM Tris–HCl, pH 7.0.

### 4.3. Preparation of iron-binding proteins

All iron-binding proteins, including bLf (from bovine milk), bovine transferrin (bTf), hLf and hTf, were purchased from Sigma in the most iron-free form available. Iron-saturated proteins and apoproteins were prepared by methods described previously [37].

### 4.4. Preparation of $^{125}$I-labeled bLf

Bovine Lf was iodinated by the lactoperoxidase method [38]. Approximately 70 mg of bLf (33% iron-saturated) was used for iodination; $^{125}$I-labeled protein was separated from free $Na^{125}I$ by chromatography on a Sephadex G-25 column. The labeled protein was aliquoted and stored at −70 °C until use. Lactoperoxidase was purchased from Boehringer-Mannheim (Montreal, Quebec), $Na^{125}I$ from Amersham (Oakville, Ontario).

### 4.5. Lactoferrin binding assay

Binding assays were performed as described [10]. Bacterial cells were harvested from culture media, washed once in PBS and resuspended in PBS containing 1% bovine serum albumin (PBS-1% BSA) to a density of $10^{10}$ cells/ml. To determine the saturation kinetics, $10^9$ bacteria (in 0.1 ml of PBS-1% BSA) were mixed and incubated with 0.1 ml of $^{125}$I-bLF solution (6.9 nM in PBS-1% BSA) for periods of 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 min at room temperature. Bacteria were pelleted and washed three times with 1 ml of ice-cold PBS containing 0.1% Tween 20. Radioactivity bound to the bacterial pellet was measured in a gamma counter. In competitive binding experiments, $10^9$ bacteria were mixed with $2 \times 10^5$ cpm $^{125}$I-bLf in the presence of serially diluted unlabeled bLf and incubated at room temperature for 2 h. Total input, cell bound and the free protein was calculated and subjected to Scatchard analysis [19]. When evaluating the inhibitory effect of bLf, apo-bLf, bTf, hLf and hTf on $^{125}$I-bLf binding, the unlabeled proteins were used at concentration of 5.5 mM. All samples were tested in triplicate, and each experiment was performed at least twice. The data presented are the means of two independent experiments (unless otherwise stated).

### 4.6. Proteolytic and heat treatment of S. uberis

Bacterial cells were treated with proteases at 37 °C for 2 h. Trypsin (Sigma) hydrolysis was performed in 100 mM Tris–Cl (pH 8.0), with a final enzyme concentration of 2500 U/ml, and the reaction was stopped by addition of phenylmethylsulfonyl fluoride (PMSF; 500 μg/ml). Pepsin (Sigma) digestion was performed in 100 mM sodium acetate buffer (pH 4.5), with an enzyme concentration of 1000 U/ml, and the pH of the reaction mix was raised to 7.4 to stop the hydrolysis. Proteinase K (Boehringer-Mannheim, Canada, Montreal, Quebec) treatment was carried out in 40 mM potassium phosphate buffer (pH 7.5), and the digestion was inhibited by the addition of PMSF (500 μg/ml). For heat treatment, the bacterial suspension was incubated in water bath for 1 h at each of the following temperatures: 50, 80 and 100 °C. Both enzyme- and heat-treated cells were washed once in PBS and resuspended in PBS-1% BSA prior to binding experiments.

### 4.7. Preparation of antiserum

Serum against recombinant Lbp was raised in rabbits by subcutaneous injection of 0.5 ml of TCA-precipitated culture supernatant from recombinant *E. coli* in complete Freund's adjuvant. Two subcutaneous boosts with the same amount of sample in incomplete Freund's adjuvant were given to each animal. The protocol for immunization was approved by the University of Saskatchewan Committee for Animal Care and all animals were housed and handled under conditions established by the Canadian Council for Animal Care.

### 4.8. Polyacrylamide gel electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed using the method described by Laemmli [39]. Samples were solubilized at 37 °C for 30 min in the absence of 2-mercaptoethanol (non-reducing conditions) or at 100 °C for 5 min in the presence of 1% 2-mercaptoethanol (reducing conditions). Samples dissolved in sample buffer with 3 M urea were boiled for 30 min prior to electrophoresis. Proteins were electrophoretically

transferred to nitrocellulose membranes as recommended by supplier (Bio-Rad) and blocked with TBS (100 mM Tris–Cl, pH 7.0, 150 mM NaCl) containing 1% BSA. To identify Lbp(s), membranes were probed with $^{125}$I-bLf at a final concentration of 80 ng/ml in TBS-1% BSA and incubated at room temperature for 2 h. After three washes with TBS containing 0.05% Tween 20, the membrane was exposed to X-ray film for 24 h at room temperature. During competition experiments, the membrane was incubated with 35 mg/ml of unlabeled bLf for 2 h before incubation with $^{125}$I-bLf.

For immunoblotting experiments, membranes were incubated with rabbit antiserum against recombinant Lbp. The second antibody used was the goat anti-rabbit IgG coupled to alkaline phosphatase (AP) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland).

### 4.9. Recombinant DNA techniques

Plasmid DNA was purified as described [40]. When required, DNA fragments were isolated from agarose gels using the Gene Clean kit (Bio/can Scientific, Mississauga, Ontario). A gene library for *S. uberis* strain Su-1 was constructed in pTZ18R [32] as described [16]. To identify bLf-binding clones, *E. coli* DH5α transformants were replica plated onto nitrocellulose disks (Schleicher and Schuel, Keene, NH) and lysed in chloroform vapor. Non-specific binding was blocked by incubation with TBS-1% BSA. Membranes were further incubated with $^{125}$I-bLf as described above. Restriction endonucleases, T4 DNA ligase, DNA polymerase I Klenow fragment and calf intestinal AP were supplied by (Pharmacia Canada Ltd, Quebec, Canada), and utilized according to the manufacturer's directions. DNA sequencing was carried out using the dideoxy-chain termination method [41] using double-stranded plasmid templates as recommended by the manufacturer (Pharmacia Canada Ltd). The sequence was determined using a combination of primers directed against both vector and insert sequences. Southern blot analysis was carried out as described [40].

### 4.10. Generation of an Lbp-deficient mutant

A deletion of the central portion of the *lbp* gene was constructed by PCR-amplification of the upstream portion and 5′-coding region and the 3′-coding sequence plus downstream region, in two separate reactions using the primers *lbp*#01 (5′-aaagtcgaccctaatatggaccaagaatcggt-3′) and *lbp*#03 (5′-ttttggtaccttaagctgtcccgc-3′), and *lbp*#02 (5′-gaggtcgacggtatcgataagc-3′) and *lbp*#04 (5′-aaaaaggtacccgccggcaaacgcaaaaaa-3′), respectively. A stop codon was included in the amplified 5′ region. PCR fragments were digested with *Kpn*I (bold type in primer sequences above), ligated, and reamplified with *lbp*#01 and *lbp*#02. The resulting PCR product was blunt-ended using the PCR Polishing Kit (Stratagene, Cedar Creek, TX), and cloned into *Sma*I digested pGh9ΔK, a derivative of pG$^+$host 9 lacking the *Kpn*I restriction endonucleases site. The resulting construct was designated pMF112a. The *aad9* spectinomycin resistance gene [42] was amplified with the primers *aad9*#01 (5′-tcgatagcttgcatgcctgcag-3′) and *aad9*#02 (5′-gaggtcgacggtatcgataagc-3′), blunt-ended and cloned into *Kpn*I-digested, blunt-ended pMF112a. The resulting plasmid was designated pMF113a and was used for allele replacement of wild type *lbp* gene. Su-1 was transformed with pMF113a, and allele-replacement of the wild type *lbp* gene was performed as described previously [43]. The allele replacement mutant was designated SuM13 and the deletion of the internal *lbp* coding sequence was confirmed by Southern blotting. The Lbp$^-$ phenotype of SuM13 was confirmed by immunoblotting using rabbit anti-Lbp polyclonal serum. AP-conjugated goat anti-rabbit IgG (Kirkegaard, and Perry Laboratories) was used as the secondary antibody. In alternative assays, bLf (Sigma) was labeled with digoxigenin using the DIG Protein Labelling Kit (Roche Diagnostics, Laval, Quebec) and was used in place of the primary rabbit anti-Lbp serum (above). Bound lactoferrin was detected using AP-conjugated anti-DIG monoclonal antibodies (Roche).

### 4.11. Adhesion assays

The ability of Su-1 and SuM13 to adhere to MAC-T MLH17 bovine mammary gland epithelial cells (ATCC Cell Line CRL-10274) was determined. MAC-T cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Sigma Chemical Co., St Louis, Missouri) as described [44], harvested, and washed in TBS. Bacteria were labeled with the PKH-2 Fluorescent Cell Linker kit (Sigma), and incubated with MAC-T cells in TBS at 37 °C for 1 h. Bacteria/host cell ratios of 10:1–100:1 were used, and bLf was added to cell mixtures at concentrations from 0.001 to 1.0 mg/ml. The level and percentage of fluorescent MAC-T cells was determined using a FACS-can flow cytometer. Assays were also carried out using unlabelled bacteria, in which case cells were examined microscopically. Cells extracted from fresh bovine mammary gland tissue [45] were also incubated with labeled bacteria, as above, and processed as above. Finally, 3 μm diameter latex beads (Sigma) were coated with recombinant Lbp and tested for their ability to adhere to MAC-T cells as described above.

### 4.12. Nucleotide sequence accession number

The nucleotide sequences reported in this manuscript were submitted to GenBank under the accession number Ay376838 for the *lbp* gene and XXXX for the *mga* gene.

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